JOURNAL OF VIROLOGY, Nov. 2001, p. 10488–10492 Vol. 75, No. 21
0022-538X/01/$04.00+0 DOI: 10.1128/JVI.75.21.10488–10492.2001

Epstein-Barr Virus and the Somatic Hypermutation of Immunoglobulin Genes in Burkitt's Lymphoma Cells

REUBEN S. HARRIS,[1]* DEBBIE S. G. CROOM-CARTER,[2] ALAN B. RICKINSON,[2] AND MICHAEL S. NEUBERGER[1]

MRC Laboratory of Molecular Biology, Cambridge CB2 2QH,[1] and CRC Institute for Cancer Studies, University of Birmingham, Birmingham B15 2TA,[2] United Kingdom

Received 21 May 2001/Accepted 25 July 2001

It has been suggested that Epstein-Barr virus (EBV) might suppress antibody maturation either by facilitating bypass of the germinal center reaction or by inhibiting hypermutation directly. However, by infecting the Burkitt's lymphoma (BL) cell line Ramos, which hypermutates constitutively and can be considered a transformed analogue of a germinal center B cell, with EBV as well as by transfecting it with selected EBV latency genes, we demonstrate that expression of EBV gene products does not lead to an inhibition of hypermutation. Moreover, we have identified two natural EBV-positive BL cell lines (ELI-BL and BL16) that hypermutate constitutively. Thus, contrary to expectations, EBV gene products do not appear to affect somatic hypermutation.

Epstein-Barr virus (EBV) is a nearly ubiquitous human gammaherpesvirus which possesses growth-transforming capabilities and which naturally infects and persists within the immune system's B-cell compartment. Viral infection can be studied in infectious mononucleosis patients, where the virus is detectable in blood and lymphoid tissues, notably tonsils. Many infected B cells express the full viral latency gene repertoire seen in EBV-transformed B lymphoblastoid cell lines (LCLs) in vitro, including six EBV-determined nuclear antigens (EBNA1, -2, -3A, -3B, -3C, and -LP), three latent membrane proteins (LMP1, -2A, and -2B), and several highly expressed, nonpolyadenylated RNAs (EBER and Bam A RNAs) (14). However, distinct, more restricted latency gene expression patterns have been detected (2, 3, 7) and may contribute significantly to lifelong viral persistence.

Antibodies provide a valuable line of defense against bacteria, parasites, and viruses. Much of this defense's strength is afforded by the sheer size of the B-cell-encoded antibody repertoire, which is created by two distinct processes. First, V(D)J recombination imprecisely juxtaposes the gene segments encoding the immunoglobulin (Ig) variable (V) region, thereby creating a large primary antibody repertoire. Second, in response to antigen, the primary repertoire is further diversified by somatic hypermutation, which, coupled with selection, produces a pool of antibodies that bind with high affinity to encountered antigens. Somatic hypermutation is largely restricted to Ig gene V regions and occurs primarily during a narrow window of B-cell development in germinal centers. Antigen-specific B cells can develop further into antibody-secreting plasma cells or long-lived memory cells poised for subsequent immune challenges.

Two lines of investigation have suggested that EBV might be capable of preventing B cells from mutating their Ig V genes. First, EBV-positive cells from infectious mononucleosis patients, although present at a high frequency within tonsils, are found rarely in tonsillar germinal centers, suggesting that this stage of development is either bypassed or inhibited (12). In support of this, mice expressing LMP1 lack obvious germinal centers, a phenotype attributable to perturbed signaling by CD40, a B-cell surface receptor required for germinal center formation (9, 22). Second, a study recently published by Kurth and colleagues classified individual tonsillar B cells with respect to expressed EBV latency genes and Ig V region DNA sequences, from which both the cellular differentiation stages and dynastic relationships could be inferred (10). This approach revealed evidence of preferential ongoing somatic hypermutation in EBV-negative as opposed to EBV-positive tonsillar B cells, which suggested that EBV might possess the capacity to stop Ig gene somatic hypermutation directly (10). Consistent with this, Denépoux and coworkers were able to induce somatic hypermutation in two EBV-negative BL cell lines (BL2 and BL45) but not in an EBV-positive BL cell line (BL74) (4).

Reasoning that a molecular understanding of the apparent immutability of EBV-positive B cells could provide key insights into an important facet of EBV biology and also an entry point that could be exploited to investigate the somatic hypermutation mechanism, we undertook experiments designed to test specifically whether EBV gene products can indeed suppress Ig V gene somatic hypermutation.

Selected EBV latency protein expression in Ramos has no effect on hypermutation. To test whether EBV latency gene products directly suppress Ig gene hypermutation, we transfected Ramos (an EBV-negative [8], constitutively hypermutating [19] BL cell line) with puromycin-resistant constructs expressing EBNA1, the only latency protein expressed ubiquitously in latently EBV-infected cells and a plausible candidate because it is the sole viral protein required for latent replication of the EBV genome (25) and therefore must recruit cellular factors for efficient DNA replication (e.g., human single-strand binding protein [hSSB] [27]), and EBNA-LP (1), an

* Corresponding author. Mailing address: MRC Laboratory of Molecular Biology, Protein and Nucleic Acid Division, Hills Rd., Cambridge CB2 2QH, United Kingdom. Phone: 44 1223 402460. Fax: 44 1223 412178. E-mail: rsharris@mrc-lmb.cam.ac.uk.

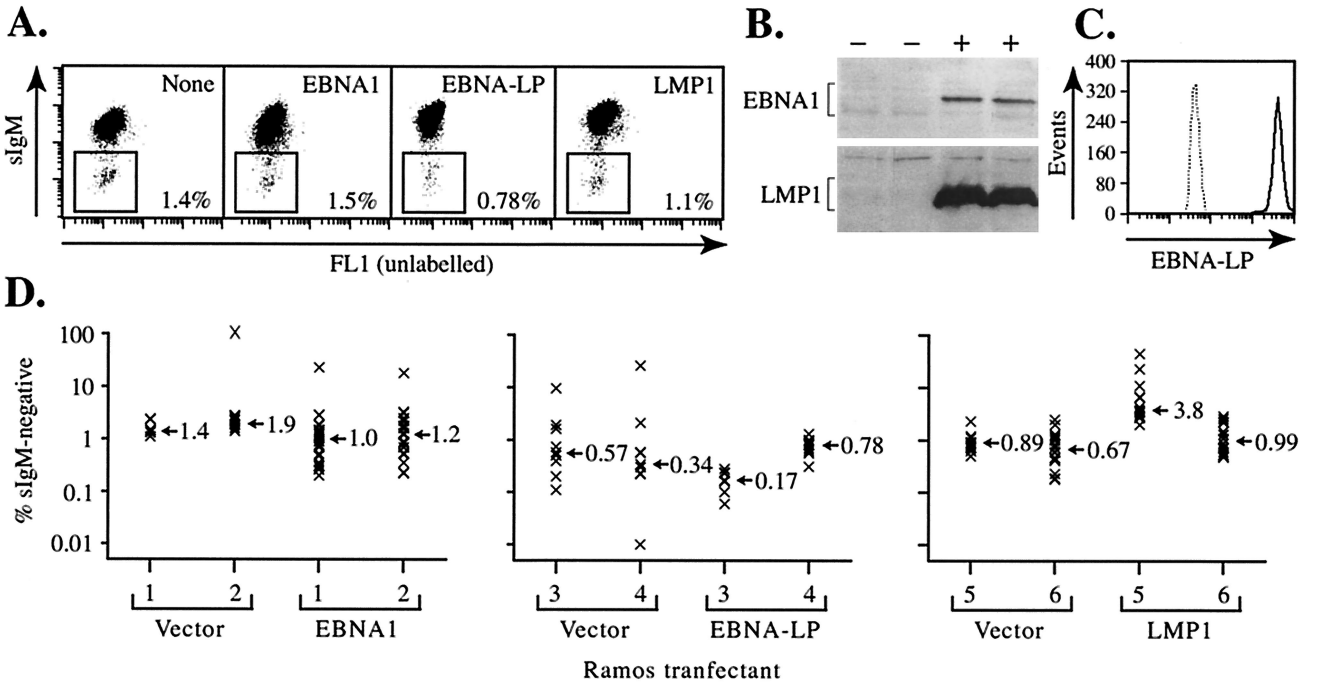

FIG. 1. Expression of selected EBV latency proteins, EBNA1, EBNA-LP, and LMP1, has little effect on Ramos hypermutation. (A) sIgM expression profiles of representative Ramos transfectants expressing vector genes alone (None), EBNA1, EBNA-LP, or LMP1. The sIgM-negative cell population is boxed, and the percentage of cells therein is indicated. Each dot represents one cell. (B) Western analysis of expression of EBNA1 (72 kDa) and LMP1 (63 kDa) in transfected Ramos subclones using monoclonal antibodies CS.1-4 (6) and 1H4 (16), respectively. (C) Cytofluorimetric confirmation of EBNA-LP expression in Ramos transfectants. Solid and dotted lines represent cells stained with an antibody specific for EBNA-LP (JF186 [5]) or a control antibody, respectively. (D) Fluctuation analyses of the sIgM-negative cell populations generated during continuous culture of subclones of Ramos transfected with empty, EBNA1, EBNA-LP, or LMP1 expression constructs. Each fluctuation analysis was performed with 9 to 23 subclones per independent transfectant. Each cross represents the percentage of single-subclone-derived cells falling within the sIgM-negative window; median percentages are indicated. Fluctuation analyses were used to assess the frequency of generation of sIgM-negative variants because a high prevalence of sIgM-negative variants in a single-cell-derived population does not itself distinguish between a high mutation frequency and an infrequent but early generation of sIgM-negative variants during clonal expansion. For example, the odd case of mostly sIgM-negative cells is presumably due to expansion of an originally sIgM-negative cell.

early-expressed coregulator of transcription and therefore also a reasonable candidate (13, 21). Ramos was also transfected with a construct expressing LMP1 (11), a presumed negative control but, as mentioned above, possibly interesting. If one of these candidate EBV gene products was capable of suppressing hypermutation, transfectants expressing it presumably would cease ongoing V_H and V_L mutation. This phenotype can be assayed by staining cells with R-phycoerythrin-conjugated goat anti-human IgM (μ chain specific; Sigma) and measuring the generation of surface IgM (sIgM)-negative variants by flow cytometry (FACSCalibur and CellQuest; Becton Dickinson); such sIgM-negative variants in the parental cell line Ramos are attributable mostly to the frequent generation of stop codons in the Ig V_H domains by hypermutation (19).

Ramos transfectants expressing each selected latency protein were established by electroporation (300 V, 950 μF; Bio-Rad Gene Pulser II), selected in medium containing 2 μg of puromycin (Sigma) per ml, continuously cultured for at least 1 month, and analyzed for the generation of sIgM-negative variants. Compared to Ramos transfected with vector only, cells expressing either EBNA1 or EBNA-LP generated similar median percentages of sIgM-negative variants, indicating that the hypermutation program remained intact (Fig. 1A and D). Ramos transfectants expressing LMP1 also displayed normal levels of sIgM-negative variants (Fig. 1A and D). Latency protein expression was confirmed by immunofluorescence microscopy of methanol-fixed cells (EBNA1 and EBNA-LP), Western blotting (EBNA1 and LMP1) (Fig. 1B), and flow cytometric analysis of paraformaldehyde-fixed NP-40-permeablized cells (EBNA-LP) (Fig. 1C). Thus, expression of EBNA1, EBNA-LP, or LMP1 alone appeared insufficient to block hypermutation.

Infection of Ramos with EBV has no effect on its mutability. To investigate whether other EBV gene products (such as EBER RNAs, Bam A RNAs, or other latency proteins) might abrogate hypermutation or whether specific latency gene products might act in concert to do so, we analyzed Ramos derivatives generated by de novo infection with EBV-neo, a derivative of the Akata type 1 EBV containing a selectable neomycin cassette (20). High-titer virus prepared and generously provided by C. Dawson (University of Birmingham) was used to infect Ramos essentially as described previously (20); infectants were selected in medium containing 2 mg of Geneticin per ml and confirmed by EBNA1 Southern hybridization. Analysis of two representative infectants, EBV1 and EBV19, revealed that, contrary to expectations, they also generated significant numbers of sIgM-negative variants, implying that the capacity for ongoing hypermutation was unaffected. Sub-

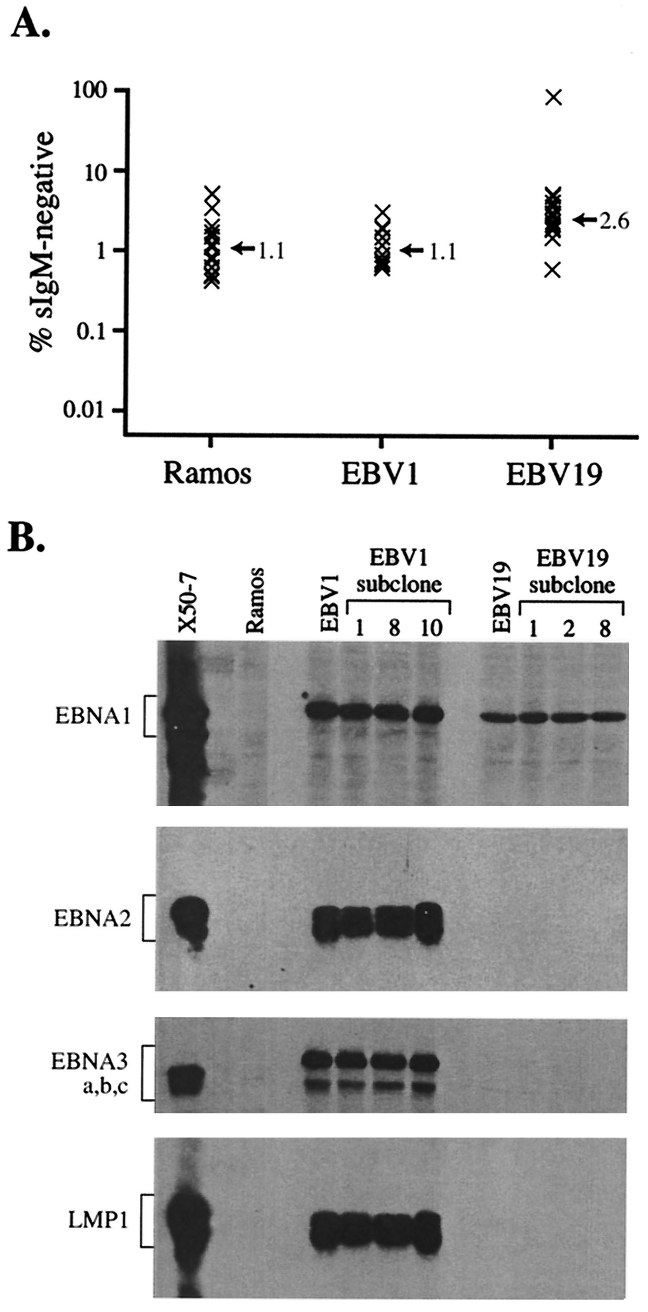

FIG. 2. Hypermutation continues unabated in Ramos newly infected with EBV. (A) Fluctuation analyses of the sIgM-negative populations generated during outgrowth of Ramos subclones ($n = 16$) or of subclones of its EBV-positive derivatives EBV1 ($n = 12$) and EBV19 ($n = 16$). Labeling is as in Fig. 1D. (B) Western analyses of EBV latency proteins expressed in positive (X50-7 [24]) and negative (Ramos) controls, EBV1, EBV19, and representative subclones thereof which were used for panel A.

cloning and fluctuation analyses demonstrated that there was indeed no apparent difference in the frequencies of sIgM-negative variants generated (Fig. 2A). Twenty-one other new infectants also produced sIgM-negative variants (data not shown).

Wondering whether the apparently intact hypermutation programs of EBV1 and EBV19 could be attributed to expression of a restricted (or nonexistent) latency gene repertoire, we probed extracts prepared from EBV1 and EBV19 with latency protein-specific antibodies. In addition to the aforementioned primary antisera, PE2 (anti-EBNA2 [26]) and HG/RS22 (human sera which recognize type 1 EBV EBNA3A-C [18]) were used. In most instances, infection of an EBV-negative BL cell line such as Ramos results in the establishment of a viral latency in which only EBNA1 and the noncoding RNAs are expressed (23). EBV19 displayed, as expected, this type of latency profile in which only EBNA1 was found (Fig. 2B). However, EBV1 defined another class of Ramos infectant, more LCL-like, in which nearly all latency genes were expressed, including EBNA3A-C (Fig. 2B). Thus, expression of neither a restricted nor a complete latency gene repertoire via de novo EBV infection seemed to affect hypermutation in Ramos.

Some naturally EBV-positive BL cell lines also mutate constitutively. Given that hypermutation proceeded normally in Ramos infected with EBV as well as in the presence of selected EBV gene products, we wondered whether it would be possible to identify naturally occurring EBV-positive B-cell lymphomas that mutate their Ig genes constitutively during culture. A survey of naturally occurring EBV-positive BL cell lines revealed an absence of a clearly identifiable population of sIgM-negative variants among many of them (e.g., Akata, BL74, Chep, Daudi, Raji, and Wan). However, a clear sIgM-negative population was noted in two of these EBV-positive cell lines, ELI-BL and BL16, suggesting an intrinsic hypermutation capacity (Fig. 3A). ELI-BL harbors a type 2 EBV, resembles germinal center B cells, and expresses a latency gene repertoire consisting only of EBNA1 and the noncoding EBER and Bam A RNAs (17). BL16 also contains a type 2 virus, but, in contrast to ELI-BL, it appears more LCL-like and expresses a full latency gene repertoire (references 15 and 17 and data not shown).

Although a clear sIgM-negative population was visible in ELI-BL and BL16 cultures, it was important to address whether these could be attributed to bonafide hypermutation. Fluctuation analysis of ELI-BL subclones revealed that the sIgM-negative variants were indeed being generated at high frequency during in vitro culture (Fig. 3B), and V_H sequence analysis (using protocols defined previously [19]) in the case of BL16 subclones confirmed that this instability reflected somatic hypermutation (Fig. 3C). Considerable V_H sequence diversity, including several sequences with multiple base substitution mutations, and an overall high V_H mutation frequency of 0.0014 mutation per base pair indicated that hypermutation is ongoing in BL16. Moreover, despite the relatively small number of V_H sequences sampled, one dynastic relationship could be inferred (first mutation at Gly54 [GGT-GAT] and second mutation at Val92 [GTG-ATG]). Finally, like in Ramos, most of the BL16 V_H base substitution mutations occurred at G or C nucleotides (24 of 33 [73%]) and clustered within the complementarity-determining regions (Fig. 3C). Thus, several hallmarks of ongoing hypermutation were also distinguishable in two natural EBV-positive BL cell lines, one expressing a limited and the other expressing a full latency gene repertoire. It was therefore clear that somatic hypermutation could proceed unabated in the presence of EBV.

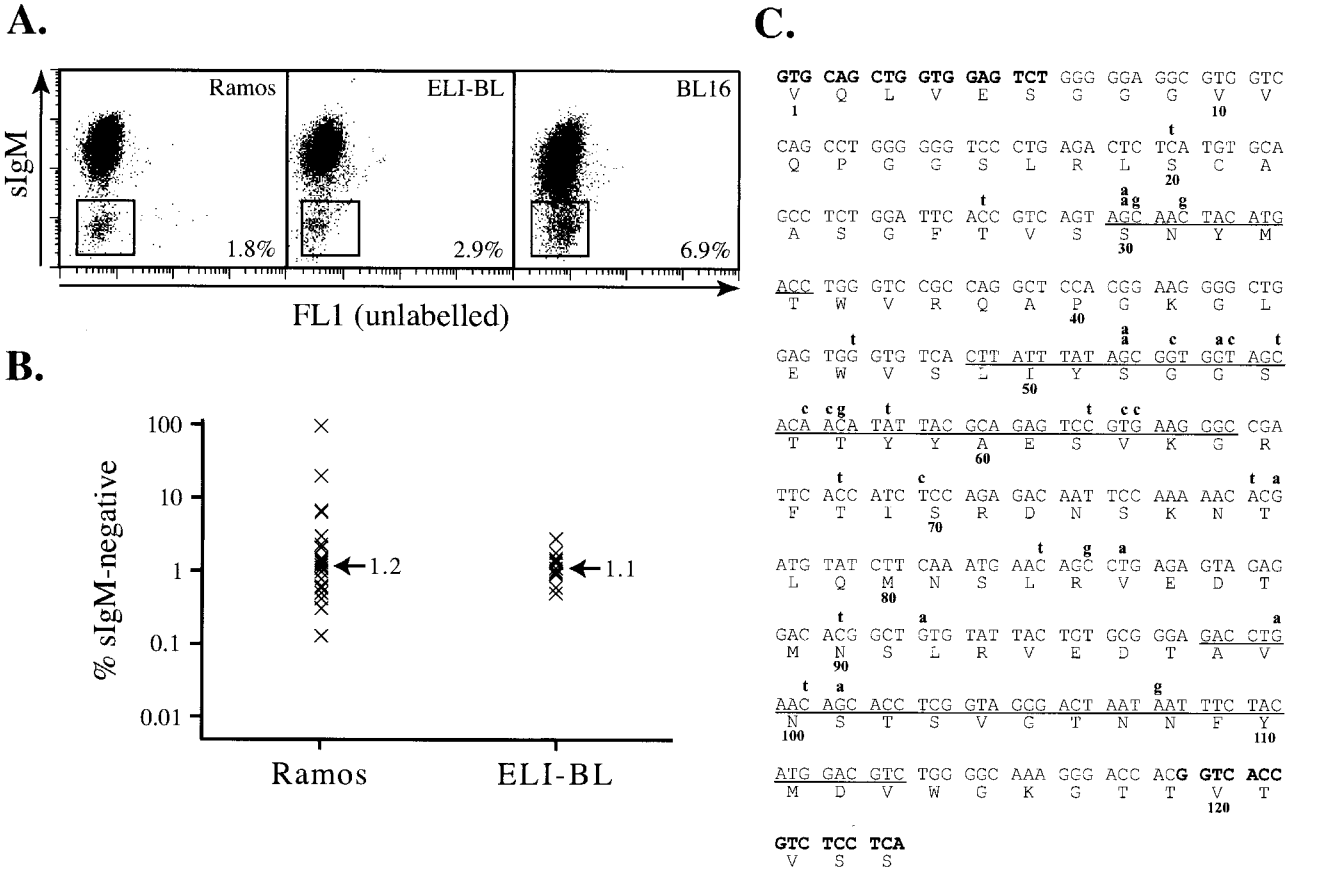

FIG. 3. Natural EBV-infected BL cell lines ELI-BL and BL16 show constitutive hypermutation. (A) sIgM expression profiles of Ramos, ELI-BL, and BL16. Labeling is as in Fig. 1A. Note that the sizeable sIgM-negative population in BL16 is in part due to less intensely staining positive cells, which also interfered with fluctuation analyses. (B) Fluctuation analyses of the sIgM-negative populations generated during outgrowth of Ramos ($n = 23$) and ELI-BL ($n = 16$) subclones. Symbols are as in Fig. 1D. (C) BL16 V_H sequence data from four independent subclones. Base substitution mutations are indicated in lowercase letters above the 338-bp consensus DNA sequence, which is in triplets of capital letters. Complementarity-determining regions and PCR primer sequences are underlined and in boldface, respectively. The corresponding amino acid sequence is indicated by single capital letters. This consensus differs at two positions from GenBank entry gi.2253343 (TCA [Ser20]-TCT and AGC [Ser55]-ACC [Thr]).

It is clearly interesting, in the light of our results, to reconsider the lack of ongoing hypermutation in EBV-positive tonsillar B cells shown by Kurth et al. (10). One possibility is that this lack of mutation does not reflect a direct association with the continued presence of EBV in these B cells but is a consequence of other pathological changes associated with infectious mononucleosis. Thus, for example, the lack of ongoing Ig V gene hypermutation in these cells could be simply because B-cell clonal expansion has occurred at a memory (postgerminal center) stage. Alternatively, there could be some circumstances in which EBV gene products might suppress hypermutation, but these remain to be defined.

Although earlier screens for hypermutating B-cell lines suggested that only EBV-negative cell lines might be capable of constitutive hypermutation (4, 19), the work described here reveals that EBV-positive BL cell lines can be also highly proficient. That this was not observed before may be due to the significant heterogeneity in BL cell line hypermutability reported here for EBV-positive lines and previously for EBV-negative cell lines (19).

A bonus from these studies has been the identification of two constitutively hypermutating human B-cell lines (ELI-BL and BL16), both of which are EBV positive. The only BL cell line previously shown to perform constitutive hypermutation was Ramos (19). The pattern of hypermutation performed in BL16 appears to be very similar to that in Ramos. Such G/C-targeted mutations are likely to constitute one part of the hypermutation program executed by human B cells in vivo (19). The results also suggest that ongoing Ig V gene hypermutation may not be such a rare attribute among BL cell lines. This identification enables a comparison of a wider panel of mutating and nonmutating BL cell lines and should facilitate further advances in understanding the molecular mechanism of somatic hypermutation.

We thank members of the Rickinson laboratory for providing valuable EBV reagents, Victoria Robinson and Lawrence Young for the EBNA1 expression construct, Julian Sale for an introduction to the Ramos system, Andy Johnson for cell sorting, and Mats Bemark, Julian Sale, and Cristina Rada for helpful commentary.

R.S.H. is a recipient of a Burroughs Wellcome Fund Hitchings-

Elion Fellowship and is a Research Fellow of Sidney Sussex College, Cambridge University.

REFERENCES

1. **Abbot, S. D., M. Rowe, K. Cadwallader, A. Ricksten, J. Gordon, F. Wang, L. Rymo, and A. B. Rickinson.** 1990. Epstein-Barr virus nuclear antigen 2 induces expression of the virus-encoded latent membrane protein. J. Virol. **64:**2126–2134.
2. **Babcock, G. J., D. Hochberg, and D. A. Thorley-Lawson.** 2000. The expression pattern of Epstein-Barr virus latent genes in vivo is dependent upon the differentiation stage of the infected B cell. Immunity **13:**497–506.
3. **Babcock, G. J., and D. A. Thorley-Lawson.** 2000. Tonsillar memory B cells, latently infected with Epstein-Barr virus, express the restricted pattern of latent genes previously found only in Epstein-Barr virus-associated tumors. Proc. Natl. Acad. Sci. USA **97:**12250–12255.
4. **Denépoux, S., N. Fournier, C. Péronne, J. Banchereau, and S. Lebecque.** 2000. T cells can induce somatic mutation in B cell receptor-engaged BL2 Burkitt's lymphoma cells independently of CD40-CD40 ligand interactions. J. Immunol. **164:**1306–1313.
5. **Finke, J., M. Rowe, B. Kallin, I. Ernberg, A. Rosen, J. Dillner, and G. Klein.** 1987. Monoclonal and polyclonal antibodies against Epstein-Barr virus nuclear antigen 5 (EBNA-5) detect multiple protein species in Burkitt's lymphoma and lymphoblastoid cell lines. J. Virol. **61:**3870–3878.
6. **Grässer, F. A., P. G. Murray, E. Kremmer, K. Klein, K. Remberger, W. Feiden, G. Reynolds, G. Niedobitek, L. S. Young, and N. Mueller-Lantzsch.** 1994. Monoclonal antibodies directed against the Epstein-Barr virus-encoded nuclear antigen 1 (EBNA1): immunohistologic detection of EBNA1 in the malignant cells of Hodgkin's disease. Blood **84:**3792–3798.
7. **Joseph, A. M., G. J. Babcock, and D. A. Thorley-Lawson.** 2000. Cells expressing the Epstein-Barr Virus growth program are present in and restricted to the naive B-cell subset of healthy tonsils. J. Virol. **74:**9964–9971.
8. **Klein, G., B. Giovanella, A. Westman, J. S. Stehlin, and D. Mumford.** 1975. An EBV-genome-negative cell line established from an American Burkitt lymphoma; receptor characteristics. EBV infectibility and permanent conversion into EBV-positive sublines by in vitro infection. Intervirology **5:**319–334.
9. **Kulwichit, W., R. H. Edwards, E. M. Davenport, J. F. Baskar, V. Godfrey, and N. Raab-Traub.** 1998. Expression of the Epstein-Barr virus latent membrane protein 1 induces B cell lymphoma in transgenic mice. Proc. Natl. Acad. Sci. USA **95:**11963–11968.
10. **Kurth, J., T. Spieker, J. Wustrow, J. G. Strickler, M. Hansmann, K. Rajewsky, and R. Kuppers.** 2000. EBV-infected B cells in infectious mononucleosis. Viral strategies for spreading in the B cell compartment and establishing latency. Immunity **13:**485–495.
11. **Liebowitz, D., J. Mannick, K. Takada, and E. Kieff.** 1992. Phenotypes of Epstein-Barr virus LMP1 deletion mutants indicate transmembrane and amino-terminal cytoplasmic domains necessary for effects in B-lymphoma cells. J. Virol. **66:**4612–4616.
12. **Niedobitek, G., H. Herbst, L. S. Young, L. Brooks, M. G. Masucci, J. Crocker, A. B. Rickinson, and H. Stein.** 1992. Patterns of Epstein-Barr virus infection in non-neoplastic lymphoid tissue. Blood **79:**2520–2526.
13. **Nitsche, F., A. Bell, and A. Rickinson.** 1997. Epstein-Barr virus leader protein enhances EBNA-2-mediated transactivation of latent membrane protein 1 expression: a role for the W1W2 repeat domain. J. Virol. **71:**6619–6628.
14. **Rickinson, A. B., and E. Kieff.** 1996. Epstein-Barr virus, p. 2397–2446. *In* B. N. Fields, D. M. Knipe, and P. M. Howley (ed.), Fields virology. Lippincott-Raven Publishers, Philadelphia, Pa.
15. **Rooney, C. M., A. B. Rickinson, D. J. Moss, G. M. Lenoir, and M. A. Epstein.** 1984. Paired Epstein-Barr virus-carrying lymphoma and lymphoblastoid cell lines from Burkitt's lymphoma patients: comparative sensitivity to non-specific and to allo-specific cytotoxic responses in vitro. Int. J. Cancer **34:**339–348.
16. **Rowe, M., H. S. Evans, L. S. Young, K. Hennessy, E. Kieff, and A. B. Rickinson.** 1987. Monoclonal antibodies to the latent membrane protein of Epstein-Barr virus reveal heterogeneity of the protein and inducible expression in virus-transformed cells. J. Gen Virol. **68:**1575–1586.
17. **Rowe, M., D. T. Rowe, C. D. Gregory, L. S. Young, P. J. Farrell, H. Rupani, and A. B. Rickinson.** 1987. Differences in B cell growth phenotype reflect novel patterns of Epstein-Barr virus latent gene expression in Burkitt's lymphoma cells. EMBO J. **6:**2743–2751.
18. **Rowe, M., L. S. Young, K. Cadwallader, L. Petti, E. Kieff, and A. B. Rickinson.** 1989. Distinction between Epstein-Barr virus type A (EBNA 2A) and type B (EBNA 2B) isolates extends to the EBNA 3 family of nuclear proteins. J. Virol. **63:**1031–1039.
19. **Sale, J. E., and M. S. Neuberger.** 1998. TdT-accessible breaks are scattered over the immunoglobulin V domain in a constitutively hypermutating B cell line. Immunity **9:**859–869.
20. **Shimizu, N., H. Yoshiyama, and K. Takada.** 1996. Clonal propagation of Epstein-Barr virus (EBV) recombinants in EBV-negative Akata cells. J. Virol. **70:**7260–7263.
21. **Sinclair, A. J., I. Palmero, G. Peters, and P. J. Farrell.** 1994. EBNA-2 and EBNA-LP cooperate to cause G0 to G1 transition during immortalization of resting human B lymphocytes by Epstein-Barr virus. EMBO J. **13:**3321–3328.
22. **Uchida, J., T. Yasui, Y. Takaoka-Shichijo, M. Muraoka, W. Kulwichit, N. Raab-Traub, and H. Kikutani.** 1999. Mimicry of CD40 signals by Epstein-Barr virus LMP1 in B lymphocyte responses. Science **286:**300–303.
23. **Wang, F., A. Marchini, and E. Kieff.** 1991. Epstein-Barr virus (EBV) recombinants: use of positive selection markers to rescue mutants in EBV-negative B-lymphoma cells. J. Virol. **65:**1701–1709.
24. **Wilson, G., and G. Miller.** 1979. Recovery of Epstein-Barr virus from nonproducer neonatal human lymphoid cell transformants. Virology **95:**351–358.
25. **Yates, J. L., N. Warren, and B. Sugden.** 1985. Stable replication of plasmids derived from Epstein-Barr virus in various mammalian cells. Nature **313:**812–815.
26. **Young, L., C. Alfieri, K. Hennessy, H. Evans, C. O'Hara, K. C. Anderson, J. Ritz, R. S. Shapiro, A. Rickinson, E. Kieff, et al.** 1989. Expression of Epstein-Barr virus transformation-associated genes in tissues of patients with EBV lymphoproliferative disease. N. Engl. J. Med. **321:**1080–1085.
27. **Zhang, D., L. Frappier, E. Gibbs, J. Hurwitz, and M. O'Donnell.** 1998. Human RPA (hSSB) interacts with EBNA1, the latent origin binding protein of Epstein-Barr virus. Nucleic Acids Res. **26:**631–637.